DESCRIPTION

RECOMBINANT ANTIBODY RECOGNIZING DIOXIN AND GENE ENCODING THE ANTIBODY

TECHNICAL FIELD

The present invention relates to a novel recombinant antibody having a binding activity to 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF), a gene encoding its amino acid sequence, a vector incorporating said gene, a transformant transformed with said vector, a process for preparing said recombinant antibody, and methods for immunologically capturing and determining 2,3,4,7,8-PeCDF using said recombinant antibody.

BACKGROUND ART

The environmental pollution by endocrine disruptors becomes problems, and research of the state of pollution, investigation of the influence on the health of human beings and the like have been carried out. As the influences of these endocrine disruptors on human beings and on the environment are revealed, they became great social concerns not only in Japan but also in many countries of the world. Among others, dioxins are suspected of lasting influences on human beings and on the ecosystem and environment, and therefore, research of the state of pollution, investigation of the state of exposure in human beings and in the ecosystem and elucidation of intake routes as well as development of a method of monitoring the amount of dioxins in polluted locations and a method of removing the pollution are accelerated. Since dioxins are formed, for example, in the course of use,

production and combustion of organic chlorine compounds, the sources of the dioxins are wide-ranging and a widespread pollution is confirmed in soil, water, atmosphere, foods, marine products and the like. Accordingly, it is desired to establish a simple and rapid method for determining dioxins in samples, owing to the need of determining dioxin concentrations in numerous samples such as biological and environmental samples, and of taking measures to the pollution.

Dioxins include a number of congeners comprised of 75 kinds of polychlorodibenzodioxins (PCDDs) and 135 kinds of polychlorodibenzofurans (PCDFs). The relative toxicity of each dioxin congener, when assuming that the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) having the highest toxicity is 1, is shown as Toxic Equivalency Factor, and 7 kinds of PCDDs and 10 kinds of PCDFs having a high toxicity are used as object substances for measurement in the analysis of dioxins. Also, of polychlorobiphenyls (PCBs) which are recognized as one group of endocrine disruptors and are seen as a problem for some time, 12 kinds of co-planar PCBs have been measured as dioxins.

Previously, the determination of dioxins has been carried out by means of a high-resolution gas chromatography/mass spectrometry (HRGC/HRMS) analysis. However, the HRGC/HRMS method needs a multi-stage of complicated cleanup procedures to remove interfering substances contained in samples, an expensive analytical instrument, and skilled analyzers. Accordingly, the use of the method is limited to an analysis in a particular analytical facility. In analytical methods of dioxins, in particular, in the RGC/HRMS method, the contents of 17

kinds of dioxin congeners each having a high toxicity value are individually quantified, and the actually quantified content of each congener is then multiplied by the corresponding Toxic Equivalency Factor. Then, the sum total of the values obtained for all the congeners is calculated as Toxic Equivalent Quantity (TEQ) which corresponds to the amount of 2,3,7,8-TCDD, and the TEQ values are used as analytical values of the dioxins. Thus, the method needs much time for analysis of samples involving data analysis. For the above reasons, it is strongly desired to develop a convenient, cheep and highly sensitive method for determining dioxins in samples.

On the other hand, there persistently exists an idea that the dioxin amount (TEQ) is more simply determined by measuring a particular indicator substance. One of these methods is that measuring chlorobenzene which is a precursor of dioxins. Recently, it has been proved that the amount of 2,3,4,7,8-PeCDF, which is one of dioxin congeners, has a very high correlation with the total TEQ value of dioxins (Takasuga et al., 11th Symposium on Environmental Chemistry, Program and Abstracts, p.136, 2002). In a wide range of samples, for example, environmental samples such as soil, mud, atmosphere, water, exhaust gas and ash, biological samples such as mother's milk and blood as well as marine products, foods and the like, 2,3,4,7,8-PeCDF is a major constituent in all dioxins, and its content shows a high correlation ($R = 0.96-0.99$) with the total TEQ value of dioxins. Accordingly, attention is paid to 2,3,4,7,8-PeCDF as an indicator substance for investigating the amount of dioxins.

On the other hand, an attempt to quantify the dioxins using an antibody is also made in the art.

For example, JP-A-2002/340882 discloses a method for determining dioxins and an apparatus therefor comprising four units, i.e. a collection unit, an extraction unit, a separation and purification unit, and a measurement unit for determining dioxins using an antibody.

Also, JP-A-2002/228660 discloses a method for detecting dioxins contained in biological samples such as human blood and mother's milk by preparing and using a monoclonal antibody having a high affinity for 2,3,7,8-TCDD.

In addition, JP-A-2002/119279 discloses a method for deducing an amount of dioxins present in samples using a few antibodies having a cross-reactivity with plural congeners within the range of dioxin.

However, these references do not disclose monoclonal antibodies recognizing 2,3,4,7,8-PeCDF, as well as, gene sequences encoding said monoclonal antibodies, recombinant antibodies based on said gene sequences and a method for determining 2,3,4,7,8-PeCDF using said recombinant antibodies.

Also, methods disclosed in these references have a disadvantage that they are insufficient for investigating the TEQ values of dioxins contained in samples.

DISCLOSURE OF THE INVENTION

(Technical problem to be solved by the invention)

The present inventors intended to establish a rapid, convenient and highly sensitive method for capturing and determining an indicator substance, 2,3,4,7,8-PeCDF by an immunological technique, the indicator substance being a major constituent among 17 kinds of dioxins determined by the HRGC/HRMS method, and the content of the indicator substance having a high

correlation with the total TEQ value of dioxins.

(Means of solving the problem)

In order to solve the above problem, the present inventors prepared two hybridomas producing monoclonal antibodies recognizing 2,3,4,7,8-PeCDF, i.e. hybridoma Dx3860r1 producing monoclonal antibody Dx3860 and hybridoma Dx3150r1 producing monoclonal antibody Dx3150, by a conventional cell fusion method using a 2,3,4,7,8-PeCDF derivative as an antigen.

Then, the present inventors isolated and purified mRNAs contained in these hybridomas, and synthesized cDNAs on the basis of the mRNAs. Subsequently, in order to select cDNAs encoding the heavy chain (H-chain) variable region and light chain (L-chain) variable region of monoclonal antibody Dx3860 as well as the H-chain variable region and L-chain variable region of monoclonal antibody Dx3150 from the synthesized cDNAs, PCR was carried out using antibody gene-specific sequences and the desired antibody genes were specifically amplified. The base sequences of the cDNAs selected were analyzed, and the amino acid sequences encoded by them were deduced.

As a result, it was found that cDNAs encoding the H-chain variable region and L-chain variable region of monoclonal antibody Dx3860 are shown by SEQ ID Nos. 1 and 2, respectively, and cDNAs encoding the H-chain variable region and L-chain variable region of monoclonal antibody Dx3150 are shown by SEQ ID Nos. 3 and 4, respectively.

Also, it was found that deduced amino acid sequences of the H-chain variable region and L-chain variable region of monoclonal antibody Dx3860 are shown by SEQ ID Nos. 5 and 6, respectively, and deduced amino acid sequences of the H-chain variable region and L-chain

variable region of monoclonal antibody Dx3150 are shown by SEQ ID Nos. 7 and 8, respectively.

In addition, the present inventors specified the amino acid sequences and positions of hypervariable domains (CDRs 1-3) in the variable regions of the above antibodies. The amino acid sequences of the hypervariable domains are shown in the following Tables 1-4.

Table 1: Amino acid sequences of the hypervariable domains in the H-chain variable region of Dx3860

CDR 1	Gly-Phe-Thr-Phe-Ser-Ser-Tyr-Ala	SEQ ID No. 9
CDR 2	Phe-Ser-Asn-Gly-Gly-Ile-Thr	SEQ ID No. 10
CDR 3	Ala-Arg-Gly-Tyr-Gly-Pro-Ala-Tyr	SEQ ID No. 11

Table 2: Amino acid sequences of the hypervariable domains in the L-chain variable region of Dx3860

CDR 1	Thr-Gly-Ala-Val-Thr-Thr-Leu-Asn-Tyr	SEQ ID No. 12
CDR 2	Asn-Thr-Asn	
CDR 3	Ala-Leu-Trp-Tyr-Ser-Asn-His-Leu	SEQ ID No. 13

Table 3: Amino acid sequences of the hypervariable domains in the H-chain variable region of Dx3150

CDR 1	Gly-Tyr-Ser-Ile-Thr-Ser-Gly-Phe-Tyr	SEQ ID No. 14
CDR 2	Ile-Ser-Tyr-Asp-Gly-Tyr-Asn	SEQ ID No. 15
CDR 3	Val-Ser-Tyr-Gly-Ser-Arg-Arg-Gly-Val-Thr-Tyr	SEQ ID No. 16

Table 4: Amino acid sequences of the hypervariable domains in the L-chain variable region of Dx3150

CDR 1	Thr-Gly-Ala-Val-Thr-Thr-Ser-Asn-Tyr	SEQ ID No. 17
CDR 2	Asn-Thr-Asn	
CDR 3	Ala-Leu-Trp-Tyr-Asn-Thr-His-Leu-Val	SEQ ID No. 18

The positions of the hypervariable domains (CDRs

1-3) in the H-chain and L-chain variable regions of monoclonal antibody Dx3860 are shown in Fig. 1 and Fig. 2, respectively, together with the DNA sequences and amino acid sequences. Also, the positions of the hypervariable domains (CDRs 1-3) in the H-chain and L-chain variable regions of monoclonal antibody Dx3150 are shown in Fig. 3 and Fig. 4, respectively, together with the DNA sequences and amino acid sequences.

In Fig. 1, positions 26-33 of the amino acid sequence represent CDR1; positions 51-57 CDR2; and positions 96-103 CDR3.

In Fig. 2, positions 26-34 of the amino acid sequence represent CDR1; positions 52-54 CDR2; and positions 91-98 CDR3.

In Fig. 3, positions 26-34 of the amino acid sequence represent CDR1; positions 52-58 CDR2; and positions 97-107 CDR3.

In Fig. 4, positions 26-34 of the amino acid sequence represent CDR1; positions 52-54 CDR2; and positions 91-99 CDR3.

Also, the present inventors integrated DNAs encoding the variable regions of the above antibodies into an expression vector, introduced the resultant vectors into host cells, and expressed recombinant antibodies in said host cells. Moreover, the present inventors confirmed that 2,3,4,7,8-PeCDF in samples can be quantified by using said recombinant antibodies. Furthermore, the present inventors introduced a mutation into DNAs encoding the variable regions of the above antibodies, expressed recombinant antibodies as described above by using the resultant mutation-introduced DNAs, and confirmed that 2,3,4,7,8-PeCDF in samples can be quantified by using said

recombinant antibodies.

Accordingly, the present invention relates to a recombinant antibody having a binding activity to 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF), which comprises at least one polypeptide selected from the group consisting of:

(1) a polypeptide constituting the H-chain variable region of monoclonal antibody Dx3860 recognizing 2,3,4,7,8-PeCDF, and having the amino acid sequence as shown in SEQ ID No. 5;

(2) a polypeptide constituting the L-chain variable region of said monoclonal antibody Dx3860, and having the amino acid sequence as shown in SEQ ID No. 6;

(3) a polypeptide constituting the H-chain variable region of monoclonal antibody Dx3150 recognizing 2,3,4,7,8-PeCDF, and having the amino acid sequence as shown in SEQ ID No. 7;

(4) a polypeptide constituting the L-chain variable region of said monoclonal antibody Dx3150, and having the amino acid sequence as shown in SEQ ID No. 8;

(5) polypeptides having amino acid sequences showing not less than 95% of homology to the amino acid sequences of the above polypeptides (1)-(4), and having a binding activity to 2,3,4,7,8-PeCDF; and

(6) polypeptides representing fragments of the above polypeptides (1)-(5), and having a binding activity to 2,3,4,7,8-PeCDF.

The present invention also relates to a DNA encoding the amino acid sequence of the above recombinant antibody, a cloning or expression vector comprising said DNA, a transformant transformed with said vector, a method for preparing said recombinant antibody using said

transformant as well as methods for immunologically capturing and determining 2,3,4,7,8-PeCDF using said recombinant antibody.

(Advantageous effects over the prior art)

It is possible to capture and determine dioxins, particularly 2,3,4,7,8-PeCDF, in a rapid, convenient and highly sensitive manner by an immunological technique using the recombinant antibody according to the present invention.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows the DNA sequence, amino acid sequence and positions of the hypervariable domains (CDRs 1-3) of the H-chain variable region of monoclonal antibody Dx3860.

Fig. 2 shows the DNA sequence, amino acid sequence and positions of the hypervariable domains (CDRs 1-3) of the L-chain variable region of monoclonal antibody Dx3860.

Fig. 3 shows the DNA sequence, amino acid sequence and positions of the hypervariable domains (CDRs 1-3) of the H-chain variable region of monoclonal antibody Dx3150.

Fig. 4 shows the DNA sequence, amino acid sequence and positions of the hypervariable domains (CDRs 1-3) of the L-chain variable region of monoclonal antibody Dx3150.

Fig. 5 shows the constitution of scFv fragment Dx3860HL.

Fig. 6 shows the constitution of scFv fragment Dx3860LH.

Fig. 7 shows the constitution of scFv fragment Dx3150HL.

Fig. 8 shows the constitution of scFv fragment Dx3150LH.

Fig. 9 represents a graph showing the results of determining 2,3,4,7,8-PeCDF by an indirect competitive immunoassay using an anti-2,3,4,7,8-PeCDF scFv.

Fig. 10 represents a graph showing the relationship of H-chain variable region polypeptide fractions with anti-2,3,4,7,8-PeCDF activity.

Fig. 11 shows the amino acid sequences of H-chain variable region mutants of monoclonal antibody Dx3860.

Fig. 12 represents a graph showing the results of determining 2,3,4,7,8-PeCDF using mutation-introduced Dx3860 scFv-displaying phages.

Fig. 13 represents a graph of comparing the antibody titers of mutation-introduced Dx3860 scFv-displaying phages.

Fig. 14 represents a graph of comparing the reactivity of mutation-introduced Dx3860 scFv-displaying phages in the presence of DMSO.

BEST MODE FOR PRACTICING THE INVENTION

In the context of the present invention, the term "antibody" includes, in addition to naturally-occurring antibodies present in the living body, polypeptides which are formed by the H-chain or L-chain variable region of an antibody or by a combination thereof and have at least one antigen-binding site. Such polypeptides include, for example, polypeptides containing only the H-chain or L-chain variable region, Fab fragments containing one set of the H-chain fragment and L-chain, $F(ab')_2$ fragments containing two sets of the H-chain fragments and L-chains, and single-chain recombinant antibodies (scFvs) in which

the H-chain variable region and L-chain variable region are linked via a linker to form a single-chain.

For example, scFvs include polypeptides containing "(H-chain variable region)-(linker)-(L-chain variable region)" in this order starting from the N-terminal side as well as polypeptides containing "(L-chain variable region)-(linker)-(H-chain variable region)" in this order starting from the N-terminal side. The linker is positioned between these regions so that the H-chain variable region and L-chain variable region are efficiently folded when the scFv binds to an antigen. The linker usually consists of 5 to 15 amino acids, and includes -$(Gly_4Ser)_3$-, for example. The linker used in the present invention is not limited by the number and type of amino acids so far as the above purpose is achieved.

Also, in the recombinant antibodies according to the present invention, further suitable amino acid sequences may be added to the N-terminal side and C-terminal side of the H-chain variable region or L-chain variable region. For example, in the case of "(H-chain variable region)-(linker)-(L-chain variable region)" polypeptides, it is possible to add a secretory signal sequence to the N-terminal side of the H-chain variable region and an epitope tag sequence to the C-terminal side of the L-chain variable region, as shown in the Examples below. Also, in the case of "(L-chain variable region)-(linker)-(H-chain variable region)" polypeptides, it is possible to add a secretory signal sequence to the N-terminal side of the L-chain variable region and an epitope tag sequence to the C-terminal side of the H-chain variable region.

The recombinant antibodies according to the

present invention include, in addition to polypeptides having at least one antigen-binding site which are formed by the H-chain or L-chain variable region of an antibody or a combination of these regions, mutated polypeptides having substantially the same functions as those of the former polypeptides. In the context of the present invention, the term "substantially the same function" means that the binding force to an antigen is substantially identical. Thus, the H-chain and L-chain variable regions of the present anti-2,3,4,7,8-PeCDF antibodies, which have the amino acid sequences as shown in SEQ ID Nos. 5-8, can contain deletion, substitution or addition mutations of one or more amino acids so far as the binding force to an antigen is substantially identical. Such a mutated polypeptide according to the present invention has preferably not less than 95%, more preferably not less than 98%, and most preferably not less than 99% of homology to the amino acid sequences as shown in SEQ ID Nos. 5-8. Also, the mutation is preferably present in a framework other than the hypervariable domains (CDRs 1-3) in antibody variable regions as shown in Figs. 1-4.

Furthermore, the recombinant antibodies according to the present invention include fragments of the polypeptides as shown in SEQ ID Nos. 5-8 which have substantially the same functions as those of the original polypeptides, and polypeptides formed by a combination of these fragments. These fragments contain at least one, preferably two and more preferably all three of the hypervariable domains (CDRs 1-3) as shown in Figs. 1-4.

The recombinant antibody according to the present invention can be produced by preparing a DNA encoding an amino acid sequence of a desired polypeptide, integrating

said DNA into an expression vector, introducing said expression vector into host cells, and cultivating said host cells in a suitable medium to express said recombinant antibody.

The DNA encoding an amino acid sequence of a desired polypeptide can be prepared by synthesis on the basis of the cDNA sequences or amino acid sequences as shown in SEQ ID Nos. 1-4 (or Figs. 1-4). Alternatively, the DNA encoding an amino acid sequence of a desired polypeptide can be obtained as follows. Thus, as shown in the Examples below, the present inventors constructed an expression vector which integrates a fragment containing the H-chain variable region of monoclonal antibody Dx3860, a linker, and the L-chain variable region of Dx3860 in this order starting from the N-terminal side (see Fig. 5), introduced the vector into Escherichia coli B, and deposited the resultant E. coli B (pET22Δ-Dx3860HL) to International Patent Organism Depositary. Also, the present inventors constructed an expression vector which integrates a fragment containing the H-chain variable region of monoclonal antibody Dx3150, a linker, and the L-chain variable region of Dx3150 in this order starting from the N-terminal side (see Fig. 7), introduced the vector into Escherichia coli K-12, and deposited the resultant E. coli K-12 (pET22Δ-Dx3150HL) to International Patent Organism Depositary. The DNA encoding an amino acid sequence of a desired polypeptide can be obtained by cleaving from these expression vectors using a suitable restriction enzyme, and optionally by introducing mutation into the resultant DNA sequence. For the purpose of ligation of DNA fragments, of course, it is possible to modify the termini of the fragments by a conventional

method.

The integration of the resultant DNA fragment into an expression vector can be carried out by tailoring the termini of the DNA fragment so as to adapt to a given fragment-insertion site of a commercially available expression vector [for example, pET-22b(+)], and inserting the terminus-tailored DNA fragment into the expression vector.

The expression vector thus obtained can be introduced into suitable host cells, particularly Escherichia coli [for example, E. coli B strain, K-12 strain, BL21(DE3) strain, etc.], and the host cells can be cultivated in a medium suitable for expression of the inserted DNA fragment to express the desired recombinant antibody. The recombinant antibody expressed can be recovered from the host cells or a culture thereof by a conventional method. The recombinant antibody recovered can be purified, for example, by a chromatography method.

Using the above process, it is possible to prepare the desired recombinant antibody at low cost and in a large amount, as compared with a monoclonal antibody obtained by cultivating animal cells in a medium in need of serum.

Using the resultant recombinant antibody, it is possible to immunologically capture 2,3,4,7,8-PeCDF in samples rapidly. Such a capturing method includes separation, purification and concentration methods of 2,3,4,7,8-PeCDF by immuno-chromatography or immuno-precipitation. Also, using the recombinant antibody and using the above action of capturing 2,3,4,7,8-PeCDF, it is possible to rapidly capture and remove 2,3,4,7,8-PeCDF which is a main substance of dioxins taken in the living

body.

Moreover, using the resultant recombinant antibody, it is possible to immunologically determine 2,3,4,7,8-PeCDF in samples in a rapid and highly sensitive manner. Such a determining method includes radioimmunoassay (RIA), enzyme immunoassay (EIA), fluoroimmunoassay (FIA) and the like.

The immunologically determining methods are classified broadly into non-competitive methods and competitive methods. The recombinant antibodies according to the present invention are preferably used for competitive methods. The competitive methods include indirect competitive methods and direct competitive methods. In the indirect competitive methods, a 2,3,4,7,8-PeCDF derivative is immobilized, and the reaction with the recombinant antibody is allowed to compete between free 2,3,4,7,8-PeCDF in samples and the immobilized antigen. In the direct competitive methods, the recombinant antibody is immobilized, and the amount of a labeled 2,3,4,7,8-PeCDF derivative is determined which binds to the immobilized recombinant antibody depending on the amount of 2,3,4,7,8-PeCDF present in samples.

EXAMPLES

The present invention is illustrated in more detail based on the following examples, but it is not limited thereto.

Preparation of hybridomas producing an anti-2,3,4,7,8-PeCDF antibody

Hybridomas producing a monoclonal antibody recognizing 2,3,4,7,8-PeCDF were prepared as follows. Thus, an alkyl chain was firstly introduced into 2,3,4,7,8-PeCDF

and the terminus of the alkyl chain was converted into an active ester. The product was then introduced into bovine serum albumin (BSA) as a carrier protein according to a conventional method to prepare a conjugate for immunization.

The conjugate for immunization was thoroughly emulsified in an adjuvant RAS R-700 (Ribi Co.). The emulsion (200 µl) was administered into the peritoneal cavity of BALB/c mice (7 weeks age, male) to immunize the mice. Booster immunizations were carried out at intervals of two weeks, blood samples were taken from the tail vein after the passage of about one week from each booster immunization, and the antibody titer in blood was determined by a competitive EIA method.

Mice in which a high level production of an antibody against 2,3,4,7,8-PeCDF was confirmed were selected and the conjugate for immunization was administered into the tail vein of the mice to carry out final immunization. After 3 to 4 days from the final immunization, the spleen was removed from the mice and spleen cells were prepared. Mouse myeloma cells (Sp2/O) in a logarithmic growth phase and the spleen cells were mixed in a ratio of 1:5, and cell fusion was carried out by a polyethylene glycol method (PEG method). The cells after fusion were suspended in a HAT medium containing 10% FCS. The suspension was pipetted into a 96-well culturing plate ($1\text{-}2.5 \times 10^5$/well), and cultivated at 37°C under 5% CO_2.

After 7 to 10 days from the onset of the cultivation, a portion of the supernatant of the culture in the wells in which proliferation of hybridomas was observed was taken and added to a microtiter plate to which the 2,3,4,7,8-PeCDF derivative-BSA conjugate was immobilized. After allowing to react at room temperature for one hour,

the plate was washed with PBS(-) containing 0.05% Tween 20. A peroxidase-labeled anti-mouse IgG antibody (recognizing γ-chain)(KPL Co.) was then added to the plate and allowed to react at room temperature for one hour, and the plate was then washed in the same manner. A substrate solution (TMB substrate, KPL Co.) was added to the plate, the peroxidase activity on the plate was measured, and the antibody titer in the supernatant of the culture was determined. Of the wells showing a high antibody titer, the wells in which the antibody titer to the immobilized 2,3,4,7,8-PeCDF derivative-BSA conjugate was largely inhibited by 2,3,4,7,8-PeCDF dissolved in 20% DMSO were selected, and the hybridomas in the wells were cloned by a limiting dilution method. Two clones producing the monoclonal antibody recognizing 2,3,4,7,8-PeCDF were established by cultivating the cells isolated by the above cloning.

Thus, hybridoma Dx3860r1 producing monoclonal antibody Dx3860 and hybridoma Dx3150r1 producing monoclonal antibody Dx3150 were obtained as described above.

Isolation and purification of mRNA

Hybridomas Dx3860r1 and Dx3150r1 producing anti-2,3,4,7,8-PeCDF antibody were grown in a RPMI 1640 medium containing 10% FCS under a 5% CO_2 aeration condition. From about $2.8\text{-}5.0 \times 10^7$ cells in a logarithmic growth phase, all RNAs were extracted by a AGPC method [Chomczynski,P., Sacchi,N., Anal.Biochem., 162, p.156-159 (1987)]. Then, poly(A)+RNA was purified using Origotex-dT 30 (latex beads to which oligo dT is bound; Takara Shuzo Co.).

Synthesis of cDNA

Using Primed first-strand reaction mix contained in Mouse scFv Module/Recombinant Phage Antibody System

(Amersham Pharmacia Co.), cDNA was synthesized from the above poly(A)+RNA. The PCR was carried out using the resultant cDNA as a template and using Mouse Ig-Primer Set (Novagen) and Taq DNA polymerase (Applied Biosystems Co.). The primer set of MuIgV_H5'-A and MuIgV_H3'-2 was used for Dx3860 H-chain, and the primer set of MuIgV_H5'-D and MuIgV_H3'-2 for Dx3150 H-chain. Also, the primer set of MuIgλV_L5'-A and MuIgλV_L3'-1 was used for both Dx3860 L-chain and Dx3150 L-chain. The primers used are shown in Table 5 below. The PCR reaction was carried out as follows. Thus, the reaction cycle of 94°C x 1 minute, 50°C x 1 minute and 72°C x 1 minute was repeated 30 cycles for Dx3860 H-chain and Dx3150 L-chain; the reaction cycle of 94°C x 1 minute, 60°C x 1 minute and 72°C x 1 minute 30 cycles for Dx3150 H-chain; and the reaction cycle of 94°C x 1 minute, 60°C x 1 minute and 72°C x 1 minute 5 cycles and then the reaction cycle of 94°C x 1 minute, 50°C x 1 minute and 72°C x 1 minute 30 cycles for Dx3860 L-chain.

Table 5: PCR primers for synthesis of cDNAs

H-chain 5'-side			
Dx3860	MuIgV_H5'-A	GGGAATTCATGRASTTSKGGYTMARCTKGRTTT	(SEQ ID No. 19)
Dx3150	MuIgV_H5'-D	ACTAGTCGACATGGRCAGRCTTACWTYYTCATTCCT	(SEQ ID No. 20)
		ACTAGTCGACATGATGGTGTTAAGTCTTCTGTACCT	(SEQ ID No. 21)
		ACTAGTCGACATGGGATGGAGCTRTATCATSYTCTT	(SEQ ID No. 22)
H-chain 3'-side (for Dx3860 and Dx3150 in common)			
MuIgV_H3'-2		CCCAAGCTTCCAGGGRCCARKGGATARACIGRTGG	(SEQ ID No. 23)
L-chain 5'-side (for Dx3860 and Dx3150 in common)			
MuIgλV_L5'-A		GGGAATTCATGGCCTGGAYTYCWCTYWTMYTCT	(SEQ ID No. 24)
L-chain 3'-side (for Dx3860 and Dx3150 in common)			
MuIgλV_L3'-1		CCCAAGCTTAGCTCYTCWGWGGAIGGYGGRAA	(SEQ ID No. 25)

Subcloning of cDNA

The above PCR products were inserted into pGEM-T

Easy using a TA cloning kit: pGEM-T Easy Vector System I (Promega Co.), and Escherichia coli XL1-Blue was then transformed using the inserts. XL1-Blue Competent Cells (STRATAGENE Co.) were used as competent cells.

Determination of base sequences and analysis of amino acid sequences

For antibody gene cDNA clones subcloned into pGEM-T Easy, sequencing reactions were carried out using T7 primer (5'-TAATACGACTCACTATAGGG: SEQ ID No. 26) and using BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Applied Biosystems Co.). Subsequently, the sequences were analyzed using ABI PRISM 310 Genetic Analyzer (Applied Biosystems Co.). As a result, base sequences of cDNAs encoding the H-chain and L-chain variable regions of the Dx3860 antibody gene and their deduced amino acid sequences (SEQ ID Nos. 1 and 2) as well as base sequences of cDNAs encoding the H-chain and L-chain variable regions of the Dx3150 antibody gene and their deduced amino acid sequences (SEQ ID Nos. 3 and 4) were obtained. The analysis of the base sequences as well as the deduction and analysis of the amino acid sequences were carried out using Analysis software DNAsis (Hitachi Soft Engineering Co.).

Also, the hypervariable domains contained in the sequences of SEQ ID Nos. 1-4 were identified according to the classification of ImMunoGeneTics data base (http://imgt.cines.fr). The data base is drawn up by referring to the articles by Lefranc, M.-P. et al. [Nucleic Acids Research, 27, p.209-212 (1999)], Ruiz, M. et al. [Nucleic Acids Research, 28, p.219-221 (2000)], and Lefranc, M.-P. [Nucleic Acids Research, 29, p.207-209 (2001)]. The positions of the hypervariable domains (CDRs 1-3) identified are shown in Figs. 1-4 together with the DNA

sequences and amino acid sequences.

Construction of expression vector pET22Δ

Expression vector pET22Δ containing a T7/lac promoter, a histidine tag and a T7 terminator was constructed by replacing the sequence between restriction enzyme sites Xba I and Nco I of a commercially available vector: pET-22b(+) (Novagen Co.) with the sequence between restriction enzyme sites Xba I and Nco I of a commercially available vector: pET-3d (Novagen Co.). The expression vector pET22Δ was cleaved with restriction enzyme Nco I (New England BioLabs Co.) and Not I (Toyobo Co.), and the termini of the resultant fragments were dephosphated using Calf intestine Alkaline Phosphatase (Toyobo Co.). The bands of pET22Δ cleaved were separated by a 0.7% agarose gel electrophoresis, gels were excised, and DNAs were extracted from the gels using MagExtractor-PCR & Gel Clean Up- (Toyobo Co.). The following scFv fragments were integrated into this Nco I-Not I site as described below and the products were used as scFv expression vectors.

Construction of scFv fragments from cDNAs

In order to link the cloned cDNAs of the H-chain and L-chain of the antibody gene via a DNA encoding a linker sequence and to integrate the product into an expression vector, the cDNAs of the H-chain and L-chain were amplified by the PCR using primers containing a sequence for a restriction enzyme,. For the Dx3150 H-chain, the primers were designed by modifying the sequence of the Bam HI site from GGATCC to GGATTC and including anterior and posterior sequences of the Bam HI site, and the amplification was carried out by dividing the H-chain DNA into two parts of 5'-side and 3'-side. In order to link the cDNAs of the H-chain and L-chain by filling-in, the

linker DNAs were amplified by the PCR using primers containing part of these sequences. By this, for the single-chain antibody in which the H-chain is located at the amino-terminal side, the sequence of the 3'-terminal side of the H-chain sense DNA was ligated to the 5'-terminal side of the linker DNA, and the sequence of the 5'-terminal side of the L-chain sense DNA was ligated to the 3'-terminal side of the linker DNA. Also, for the single-chain antibody in which the L-chain is located at the amino-terminal side, the sequence of the 3'-terminal side of the L-chain sense DNA was ligated to the 5'-terminal side of the linker DNA, and the sequence of the 5'-terminal side of the H-chain sense DNA was ligated to the 3'-terminal side of the linker DNA. The combinations of the primers used for amplification of the H-chain, L-chain and linker DNA are shown in Table 6 and Table 7.

Table 6: PCR primers for construction of scFvs

Oligo for linker (sense)	
GGA GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCC (SEQ ID No. 27)	
Dx3860 HL	
·H-chain	
3860H 5' (Nco)	G ACC ATG GAA GTG AAG CTG GTG GAG TCC GGG GG (SEQ ID No. 28)
3860H 3' (Mro)	CC TCC GGA AGA GAC AGT GAC CAG GGT ACC TTG GC (SEQ ID No. 29)
·L-chain	
3860L 5' (Bam)	GC GGA TCC CAG GCT GTT GTG ACT CAG GAA TCT (SEQ ID No. 30)
3860L 3' (Not)	G AGC GGC CGC GCC TAG GAC AGT CAG TTT GGT (SEQ ID No. 31)
·Linker extension	
Linker 5' (3860H)	GGT ACC CTG GTC ACT GTC TCT TCC GGA GGA GGC GGT TCA G (SEQ ID No. 32)
Linker 3' (3860L)	AGA TTC CTG AGT CAC AAC AGC CTG GGA TCC GCC ACC GCC AG (SEQ ID No. 33)
Dx3860 LH	
·L-chain	
3860L 5' (Nco)	G ACC ATG GCC CAG GCT GTT GTG ACT CAG GAA TCT (SEQ ID No. 34)
3860L 3' (Mro)	CC TCC GGA GCC TAG GAC AGT CAG TTT GGT TCC TCC (SEQ ID No. 35)
·H-chain	
3860H 5' (Bam)	GC GGA TCC GAA GTG AAG CTG GTG GAG TCC GGG GGA GG (SEQ ID No. 36)
3860H 3' (Not)	G AGC GGC CGC TGC AGA GAC AGT GAC CAG AGT (SEQ ID No. 37)
·Linker extension	
Linker 5' (3860L)	ACC AAA CTG ACT GTC CTA GGC TCC GGA GGA GGC GGT TCA G (SEQ ID No. 38)
Linker 3' (3860H)	CCC GGA CTC CAC CAG CTT CAC TTC GGA TCC GCC ACC GCC AG (SEQ ID No. 39)

Table 7: PCR primers for construction of scFvs (Continued)

Dx3150 HL	
·H-chain (5'-side)	
3150H 5' (Nco)	G ACC ATG GAT GTA CAG CTT CAG GAG TCA GGA CC (SEQ ID No. 40)
3150H (128 at)	CC TGG AAA CTG CCG AAT CCA GTT CCA GT (SEQ ID No. 41)
·H-chain (3'-side)	
3150H (101 sn)	AC TGG AAC TGG ATT CGG CAG TTT CCA GG (SEQ ID No. 42)
3150H 3' (Mro)	CC TCC GGA GGA GAC TGT GAG AGT GGT ACC TTG GC (SEQ ID No. 43)
·L-chain	
3150L 5' (Bam)	GC GGA TCC CAG GCT GTT GTG ACT CAG GAA TCT (SEQ ID No. 44)
3150L 3' (Not)	G AGC GGC CGC GCC TAG GAC AGT CAG TCT GGT (SEQ ID No. 45)
·Linker extension	
Linker 5' (3150H)	GGT ACC ACT CTC ACA GTC TCC TCC GGA GGA GGC GGT TCA G (SEQ ID No. 46)
Linker 3' (3150L)	AGA TTC CTG AGT CAC AAC AGC CTG GGA TCC GCC ACC GCC AG (SEQ ID No. 47)
Dx3150 LH	
·L-chain	
3150L 5' (Nco)	G ACC ATG GCC CAG GCT GTT GTG ACT CAG GAA TCT (SEQ ID No. 48)
3150L 3' (Mro)	CC TCC GGA GCC TAG GAC AGT CAG TCT GGT TCC TCC (SEQ ID No. 49)
·H-chain (5'-side)	
3150H 5' (Bam)	GC GGA TCC GAT GTA CAG CTT CAG GAG TCA GGA CCT GG (SEQ ID No. 50)
3150H (128 at)	CC TGG AAA CTG CCG AAT CCA GTT CCA GT (SEQ ID No. 51)
·H-chain (3'-side)	
3150H (101 sn)	AC TGG AAC TGG ATT CGG CAG TTT CCA GG (SEQ ID No. 52)
3150H 3' (Not)	G AGC GGC CGC TGA GGA GAC TGT GAG AGT GGT (SEQ ID No. 53)
·Linker extension	
Linker 5' (3150L)	ACC AGA CTG ACT GTC CTA GGC TCC GGA GGA GGC GGT TCA G (SEQ ID No. 54)
Linker 3' (3150H)	TCC TGA CTC CTG AAG CTG TAC ATC GGA TCC GCC ACC GCC AG (SEQ ID No. 55)

The PCR amplification was carried out using GeneAmp PCR System 9700 (Applied Biosystems Co.) and using rTaq DNA polymerase (Toyobo Co.), as follows. Thus, the reaction cycle of 94°C x 1 minute, 58°C x 1 minute and 72°C x 1 minute was repeated 5 cycles and then the reaction cycle of 94°C x 1 minute, 48°C x 1 minute and 72°C x 1 minute 20 cycles. After the PCR amplification, each PCR product was separated by a 3% agarose gel electrophoresis. A part of gel containing a DNA fragment was excised, and the DNA was extracted from the gel using MagExtractor -PCR & Gel Clean Up- (Toyobo Co.). Subsequently, the three extracted DNAs of the H-chain, L-chain and linker DNA were mixed, and the H-chain, L-chain and linker DNA were ligated together by repeating 20 cycles the reaction cycle of 94°C x 1.5 minutes and 65°C x 3 minutes using rTaq DNA polymerase (Toyobo Co.), or 20 cycles the reaction cycle of 95°C x 1.5 minutes and 65°C x 6 minutes using Pfu DNA polymerase (STRATAGENE Co.).

The scFv fragments thus ligated are shown in Figs. 5-8 together with the amino acid sequences encoded thereby (SEQ ID Nos. 56-59; or SEQ ID Nos. 60-63 for amino acid sequences only).

Fig. 5 (SEQ ID No. 56) shows the scFv fragment (Dx3860HL) containing the H-chain variable region of monoclonal antibody Dx3860, a linker, and the L-chain variable region of Dx3860 in this order starting from the N-terminal side, and positions 1-114 of the amino acid sequence shows the H-chain variable region, positions 115-129 the linker, and positions 130-239 the L-chain variable region.

Fig. 6 (SEQ ID No. 57) shows the scFv fragment (Dx3860LH) containing the L-chain variable region of

monoclonal antibody Dx3860, a linker, and the H-chain variable region of Dx3860 in this order starting from the N-terminal side, and positions 1-110 of the amino acid sequence shows the L-chain variable region, positions 112-126 the linker, and positions 127-240 the H-chain variable region.

Fig. 7 (SEQ ID No. 58) shows the scFv fragment (Dx3150HL) containing the H-chain variable region of monoclonal antibody Dx3150, a linker, and the L-chain variable region of Dx3150 in this order starting from the N-terminal side, and positions 1-118 of the amino acid sequence shows the H-chain variable region, positions 119-133 the linker, and positions 134-243 the L-chain variable region.

Fig. 8 (SEQ ID No. 59) shows the scFv fragment (Dx3150LH) containing the L-chain variable region of monoclonal antibody Dx3150, a linker, and the H-chain variable region of Dx3150 in this order starting from the N-terminal side, and positions 1-110 of the amino acid sequence shows the L-chain variable region, positions 112-126 the linker, and positions 127-244 the H-chain variable region.

Furthermore, in order to amplify the resultant scFv fragment, the PCR was carried out by adding primers corresponding to both ends (Nco I-Not I) of the scFv to the reaction solution. For Dx3860, the reaction cycle of 94°C x 1 minute, 67°C x 1 minute and 72°C x 2 minutes was repeated 5 cycles, and then the reaction cycle of 94°C x 1 minute, 60°C x 1 minute and 72°C x 2 minutes 20 cycles. For Dx3150, the reaction cycle of 95°C x 1 minute, 62°C x 1 minute and 75°C x 4 minutes was repeated 5 cycles, and then the reaction cycle of 95°C x 1 minute, 55°C x 1 minute and

75°C x 4 minutes 20 cycles. The PCR products were separated by a 1.5% agarose gel electrophoresis, a part of gel containing a DNA fragment (730-740 bp) of the scFv was excised, and the DNA fragment was extracted from the gel. Subsequently, the termini of the DNA fragment were treated with restriction enzymes Nco I (New England BioLabs Co.) and Not I(Toyobo Co.), and the fragment was again purified using MagExtractor.

The scFv DNA fragment was inserted into the Nco I-Not I site of expression vector pET22Δ, and Escherichia coli XL1-Blue was transformed with the expression vector. DNA Ligetion Kit Ver.2 (Takara Shuzo Co.) was used for ligation, and XL1-Blue Competent Cells (STRATAGENE Co.) were used as competent cells. The clones subcloned were analyzed for the sequence of scFv part, and clones having a correct sequence were selected and used for the expression of the scFv. As described above, expression vector pET22Δ-Dx3860HL containing scFv fragment Dx3860HL as well as expression vector pET22Δ-Dx3150HL containing scFv fragment Dx3150HL were obtained.

Expression vector pET22Δ-Dx3860HL was introduced into Escherichia coli B and expression vector pET22Δ-Dx3150HL was introduced into Escherichia coli K-12. The resultant Escherichia coli B (pET22Δ-Dx3860HL) and Escherichia coli K-12 (pET22Δ-Dx3150HL) were deposited to International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan) under the terms of the Budapest Treaty on February 27, 2003, and obtained the accession numbers of FERM BP-8305 and FERM BP-8306, respectively.

Expression in Escherichia coli

Escherichia coli Origami B (DE3) (Novagen Co.) transformed with expression vector pET22Δ-Dx3860HL integrating scFv fragment Dx3860HL was cultivated in a LB medium (300 ml) at 37°C until about 0.5 of OD600 was obtained. The temperature was then lowered to 25°C and the cultivation was continued. When about 1.0 of OD600 was obtained, IPTG (isopropylthiogalactoside) was added to the culture until the final concentration of 1 mM, and the cultivation was continued overnight to induce the expression of the scFv. Cell bodies (about 1.0g) were recovered by centrifugation and suspended in 50 mM tris-HCl (pH 8.0), 0.1 M NaCl. Then, lysozyme (final concentration 0.2 mg/ml) and Triton X-100 (final concentration 1%) were added to the suspension to effect the cell lysis. The precipitate was recovered by centrifugation (15,000 x g, 20 minutes) and washed twice with a buffer containing 1.0% Triton X-100 to obtain about 100 mg of the precipitate containing the scFv.

Reconstitution of scFv

The scFv obtained as an inclusion body was added to a buffer of 25 mM PB, 350 mM NaCl and 6 M guanidine-HCl (pH 7.4), and dissolved by allowing it to stand overnight at 4°C. The residue was removed by centrifugation (10,000 x g, 15 minutes), and the solution was then applied to a nickel-chelate column (Qiagen Co.) equilibrated with the above buffer. The column was thoroughly washed with the buffer in an amount of about 5-10 times of the column volume, and the buffer was then replaced with the above buffer containing 20% glycerol and 400 mM alginine. The scFv bound to the chelate column was reconstituted using a guanidine-HCl gradient of 6 M to 0 M. The column was

washed with a solution (pH 7.4) of 25 mM PB, 350 mM NaCl, 20% glycerol, and 50 mM imidazole, and the scFv was then eluted by raising the imidazole concentration to 300 mM.

Determination of 2,3,4,7,8-PeCDF by an indirect competitive immunoassay using anti-2,3,4,7,8-PeCDF scFv

One μg/ml of 2,3,4,7,8-PeCDF derivative-BSA conjugate (50 μl) was added to a microtiter plate, and allowed to react at room temperature for one hour. The wells of the microtiter plate were washed with PBS(-) containing 0.05% Tween 20. A solution of Block Ace (Yukijirushi Co.) was then added to the wells, and the wells were blocked by allowing them to stand at room temperature for two hours. After the microtiter plate was washed, a solution (25 μl) of 2,3,4,7,8-PeCDF prepared in various concentrations (a solution in 20% DMSO) and a solution (25 μl) of anti-2,3,4,7,8-PeCDF scFv were added to the wells, and allowed to react at room temperature for 0.5 to 1 hour. After the microtiter plate was again washed, a solution of anti-tetra-His antibody (Qiagen Co.) 2000-fold diluted was added to the wells, and allowed to react at room temperature for one hour. Subsequently, a solution (50 μl) of peroxidase-labeled anti-mouse IgG (recognizing γ-chain) antibody (KPL Co.) 3000-fold diluted was added to the wells, and allowed to react at room temperature for one hour. After the wells of the microtiter plate were thoroughly washed to remove an unreacted solution, a substrate solution (TMB substrate, KPL Co.) was added to the wells and the plate was allowed to stand at room temperature for 15 minutes. The reaction was terminated by adding 1 M H_3PO_4 (50 μl), and OD450 (control: OD600) was measured using a plate reader (Labsystems Co.). The results are shown in Fig. 9 as a graph. It is evident from

the graph that 2,3,4,7,8-PeCDF can be determined in a highly sensitive manner using the anti-2,3,4,7,8-PeCDF scFv.

Confirmation of 2,3,4,7,8-PeCDF binding activity of H-chain variable region polypeptide

Escherichia coli Origami B (DE3) (Novagen Co.) was transformed using expression vector pET22Δ-Dx3860H which was prepared by removing the sequence between restriction enzyme sites Bam HI and Not I containing the L-chain variable region of expression vector pET22Δ-Dx3860HL. Using the transformant, the expression of the H-chain variable region polypeptide (polypeptide having the amino acid sequence as shown in SEQ ID No. 5) was carried out in the same manner as in the case of the scFv.

The H-chain variable region polypeptide obtained as an inclusion body was reconstituted on a nickel-chelate column as described above, and the product was then isolated and purified using imidazole. Protein concentrations of the fractions eluted from the chelate column were determined by measuring absorbance (280nm), and the reactivity to an immobilized 2,3,4,7,8-PeCDF-BSA conjugate was also investigated by an EIA method. As a result, it was recognized that the fractions of the H-chain variable region polypeptide have an anti-2,3,4,7,8-PeCDF activity as shown in Fig. 10. Thus, it was confirmed that the H-chain variable region polypeptide has a binding activity to 2,3,4,7,8-PeCDF.

Introduction of mutation into H-chain and confirmation of 2,3,4,7,8-PeCDF binding activity of the mutant

A library of mutation-introduced antibody genes was prepared on the basis of the gene sequence of monoclonal antibody Dx3860. The mutation was introduced by an error-prone PCR using the gene of the H-chain variable

region (V_H) of the antibody as a template and using primers prepared by adding restriction enzyme sites to the sequences of 5'-side and 3'-side. The error-prone PCR is a method of introducing random mutations by utilizing the property of the Taq DNA polymerase often causing reading-errors during amplification and by intentionally inducing the reading-errors during the PCR by the addition of manganese chloride. The PCR products were terminus-treated with restriction enzymes and purified, and the products were substituted for the H-chain gene of a phagemid expressing a single-chain antibody using the restriction enzyme sites. The resultant phagemids were used to transform Escherichia coli TG1.

To a culture (10 ml) of E. coli transformed, ampicillin was added in a final concentration of 100 µg/ml and M13KO7 phage in a final concentration 4 x 10^9 pfu/ml, and cultivation was carried out at 37°C for one hour. The cell bodies were recovered by centrifugation, resuspended in a 2x YT medium (10 ml) containing 100 µg/ml ampicillin and 50 µg/ml kanamycin, and cultivated at 37°C overnight to produce single-chain antibody-displaying phages in the medium. The culture was centrifuged, and a 20% polyethylene glycol solution (2 ml) containing 2.5 M NaCl was added to and mixed with the supernatant (10 ml) of the culture excluding the E. coli cell bodies. The mixture was allowed to stand on ice for one hour, and then centrifuged (10000g x 20 minutes) under cooling. After the supernatant was fully removed, the remaining precipitate was dissolved in a solution (1 ml) of Block Ace (Yukijirushi Co.) 10-fold diluted, and the resultant solution was used as a solution of single-chain antibody-displaying phages.

Subsequently, biopanning was carried out in order

to concentrate clones having a high reactivity to a 2,3,4,7,8-PeCDF derivative, a 2,3,7,8-TCDF derivative, and a chlorobenzene derivative from the single-chain antibody-displaying phages prepared. The phage solution prepared was firstly preincubated in a microtiter plate to which only a blocking agent was immobilized (100 µl/well, at room temperature for one hour) to exclude a nonspecific binding. The phage solution was then transferred to a microtiter plate to which respective BSA conjugates of a 2,3,4,7,8-PeCDF derivative, a 2,3,7,8-TCDF derivative, and a chlorobenzene derivative were immobilized and which was blocked with a solution of Block Ace (100 µl/well), and allowed to react at room temperature for one hour in the presence of 8% DMSO. After the reaction, PBS(-) containing 8% DMSO and 0.1% Tween 20 (300 µl) was added to the wells of the plate, and pipetting was carried out. The plate was allowed to stand for 5 minutes, and the washing buffer was then discarded. After the washing procedure was repeated three times, the washing buffer was fully removed. Then, 0.1 M glycine-HCl buffer (pH 2.2) (100 µl/well) was added to the wells, and the plate was allowed to stand for 10 minutes. After pipetting, the single-chain antibody-displaying phages liberated from the immobilized antigens were recovered and immediately neutralized by adding a tris solution (pH 8.0).

The phage solution recovered by biopanning was mixed with a culture of E. coli TG1 (OD_{600nm} = 0.3) cultivated in a 2x YT medium (2.5 ml), and the mixture was cultivated at 37°C for one hour to effect reinfection with the phage. Subsequently, M13KO7 phage was added in a final concentration 4 x 10^9 pfu/ml to a culture containing ampicillin (final concentration 100 µg/ml) and glucose

(final concentration 2%), and further cultivation was carried out at 37°C for one hour. The cell bodies were recovered by centrifugation, resuspended in a 2x YT medium (10 ml) containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, and cultivated at 37°C overnight. By the above procedures, single-chain antibody-displaying phages were amplified and produced in a medium (phage rescue). The phages amplified were again recovered by polyethylene glycol precipitation. The concentration and reinfection by the biopanning as well as the amplification by the phage rescue were repeated three to five times.

E. coli TG1 was infected with phage clones regarded as concentrated sufficiently, plated on an agar plate, and cultivated at 30°C overnight to form single colonies. Six clones were selected at random from the single colonies of TG1 every screening conditions, phagemids were prepared by a conventional method, and the sequencing reaction with BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Applied Biosystems Co.) was carried out using the phagemids as a template. The base sequences were analyzed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems Co.), and 4 kinds of mutants having mutations in the H-chain variable region (Dx3860HL-M#5, Dx3860LH-M#1, Dx3860LH-M#2, Dx3860LH-M#3) were obtained. The amino acid sequences of these mutants having mutations in the H-chain variable region are shown in Fig. 11 and SEQ ID Nos. 64-67. In comparison with the H-chain variable region of wild-type Dx3860, 1 or 2 mutations of amino acids were observed. Furthermore, mutation-introduced positions were not limited to CDR sites but observed in the framework as well.

It was confirmed that all the mutants recognize

2,3,4,7,8-PeCDF by an indirect competitive immunoassay (Fig. 12). Also, it was found that there is a difference in the antibody titer and reactivity in DMSO and the mutants show superior results over the wild-type in both the antibody titer and stability in DMSO (Fig. 13 and Fig. 14). Moreover, the tendency was retained in each scFv expressed by E. coli Origami B (DE3) transformed.

INDUSTRIAL AVAILABILITY

It is possible to prepare a recombinant antibody recognizing 2,3,4,7,8-PeCDF in a large amount by using a DNA provided by the present invention to express the antibody in host cells. The recombinant antibody thus prepared is cheaper than the parent monoclonal antibody. The present recombinant antibody can be used for capturing 2,3,4,7,8-PeCDF immunologically and applied to an immunoassay. Also, using a DNA into which a mutation is introduced, it is possible to prepare a recombinant antibody having a further advantageous property, for example, a recombinant antibody having an improved affinity for 2,3,4,7,8-PeCDF or a recombinant antibody having an improved stability, and to solve various problems associated with naturally occurring antibody proteins.